Virology 288, 275–282 (2001)
doi:10.1006/viro.2001.1103, available online at http://www.idealibrary.com on IDEAL®

Intranuclear Localization of the Transcription Coadaptor CBP/p300 and the Transcription Factor RBP-Jk in Relation to EBNA-2 and -5 in B Lymphocytes

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Received April 17, 2001; returned to author for revision June 6, 2001; accepted July 18, 2001

We have studied the expression and the localization of the cellular proteins CBP/p300 and RBP-Jk in *in vitro* EBV-infected human B lymphocytes in relation to the EBNA-2 and EBNA-5 proteins. We found that the level of CBP/p300 was elevated drastically by EBV infection and also after activation by CD40 ligation. Thus the increase in CBP/p300 expression in the EBV-infected cells is related to the virus-induced activation and proliferation of the cells. EBNA-2 and RBP-Jk colocalized in the nucleoplasm, which is in accordance with their functional interaction. We confirmed earlier reports about the presence and colocalization of EBNA-5 and CBP in the nuclear POD bodies. On the other hand, neither EBNA-2 nor p300 was detected in the PODs. The expression of these two proteins overlapped in some distinct dots of the nucleoplasm. Taken together, the different patterns of CBP and p300 expression and their different localization in relation to the PML bodies and two EBV-encoded proteins in the B cells may provide some clue to their distinct functional roles.

Key Words: EBV, EBNA-5, EBNA-2, RBP-Jk, CBP, p300, PML bodies.

INTRODUCTION

Resting B lymphocytes can be immortalized to generate permanently growing cultures by Epstein–Barr virus (EBV) infection. The resulting lymphoblastoid cell lines (LCLs) express nine virally encoded proteins. Among these, six are located in the nucleus (EBNA-1 to -6) and three are membrane proteins (LMP-1, -2A, -2B) (reviewed by Rickinson and Kieff, 1996). EBNA-2 and EBNA-5 (also named EBNA-LP) are the earliest virally encoded proteins expressed after infection. They drive the resting B cells to enter the cell cycle (Sinclair *et al.,* 1994) and thus initiate the EBV-induced immortalization process.

EBV recombinant-based genetic analysis showed that EBNA-1, -2, -3, -5, -6, and LMP-1 are essential for B cell immortalization. EBNA-2 functions as a transactivator that upregulates both viral and cellular genes, such as LMP-1, LMP-2A, and LMP-2B, the viral C-promoter (Woisetschlaeger *et al.,* 1991), and c-myc (Kaiser *et al.,* 1999). EBNA-2 interacts with DNA through the mediation of the cellular transcription factors RBP-Jk and PU.1 (Johannsen *et al.,* 1995; Ling *et al.,* 1993). It also collaborates with the transcriptional coactivators CBP and p300 (Wang *et al.,* 2000).

EBNA-5 is important for EBV-induced B cell transformation. Recombinant EBV that carried an EBNA-5 mutant showed a 10-fold-reduced immortalizing capacity (Mannick *et al.,* 1991). EBNA-5 enhances the Cp and LMP-1 promoters activating the function of EBNA2 (Harada and Kieff, 1997; Nitsche *et al.,* 1997). In some LCLs EBNA-5 appears in the PML bodies (Székely *et al.,* 1996), which also contain pRb (Jiang *et al.,* 1991) and the 70-kDa heat shock protein (hsp 70) (Székely *et al.,* 1995).

The CREB-binding protein (CBP) and p300 are transcriptional coactivators with histone acetylase activity (Bannister and Kouzarides, 1996). CBP and p300 have 70% homology. They interact with cellular proteins such as c-jun and p53 (Bannister *et al.,* 1995; Scolnick *et al.,* 1997) and with the adenoviral protein E1A and with EBNA-2 (Arany *et al.,* 1995; Wang *et al.,* 2000). In murine embryo fibroblasts, CBP influences the EBNA-2-mediated activation of the c-myc promoter through the interferon responsive PRF element. In this system, p300 has an opposite, repressor effect (Jayachandra *et al.,* 1999).

In the present work, we have confirmed that in addition to their nucleoplasmic localization EBNA-5 and CBP are present in the PML bodies (PODs). In contrast to the finding on Hep-2 cells (von Mikecz *et al.,* 2000), we did not detect p300 in the PODs of B cells. EBNA-2 and RBP-Jk showed almost complete colocalization in the nucleoplasm. Some large dots in the nucleoplasm were positive for both EBNA-2 and p300.

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0042-6822/01 $35.00

RESULTS

Expression of CBP and p300 in tonsil-derived B cells

The expression of CBP and p300 proteins was detectable by immunofluorescence in all tonsil-derived B cells. Some large cells were present in the freshly separated B cell suspension. These disappeared after 96 h in culture. In contrast, the majority of cells were blasts after 96 h in the EBV-infected samples (Figs. 1C and 1G).

CBP staining was confined to the nucleoplasm and had a trabecular pattern (Fig. 1A). In addition, several bright dots could be distinguished which, according to an earlier description, represented PML bodies (LaMorte *et al.,* 1998). In the small resting B cells, the staining of p300 was weaker than that of CBP (Figs. 1A and 1E). p300 staining showed homogeneously distributed fine dots. In uninfected control cell cultures at 96 h, both CBP and p300 stainings were weak in all cells, but in the CBP staining brilliant dots could be still seen (Figs. 1D and 1H).

Ninety-six hours after infection the majority of cells were blast transformed, as indicated by the decondensed chromatin and the large nucleus. Compared to the small cells, the CBP staining in the nucleoplasm was more homogeneous and the number of PML bodies was higher in the blasts (Fig. 1C). They could be distinguished easily against the homogeneously distributed staining in the background. Compared to the small cells, p300 staining became conspicuously stronger in the blasts and distinct brilliant dots could be distinguished (Figs. 1F and 1G).

The CBP/p300 levels were assessed by immunoblotting (Fig. 2A). In fresh cells CBP and p300 could be detected and the amounts of both proteins decreased in the cultures. In the EBV-infected samples, they also decreased after 24 h, but in the 96-h culture their levels were considerably increased. Detection by immunofluorescence and by immunoblotting showed similar results, which are in accordance with the preponderance of blasts in the 96-h cultures. To clarify whether CBP/p300 induction is confined to the EBV-infected cells, peripheral blood B cells were activated for 96 h by exposure to CD40L. In these cultures, the majority of cells were blasts and the CBP and p300 levels increased considerably (Fig. 2B).

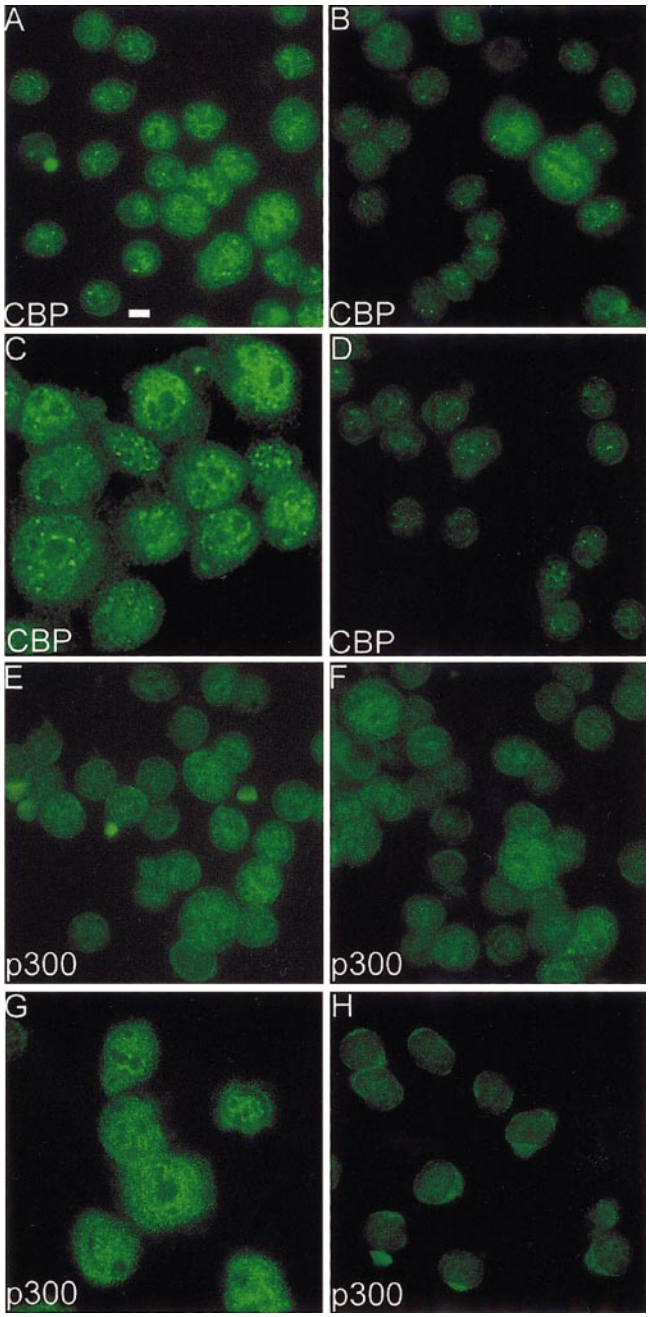


FIG. 1. Immunofluorescence staining for CBP/p300 in EBV-infected B cell populations. (A, E) 0 h, (B, F) 24 h, and (C, G) 96 h after EBV infection. (D, H) Uninfected B cells cultured for 96 h. The population contained 33 and 68% EBNA2-positive cells in the samples 24 and 96 h after infection, respectively. Note the enlargement of the cells 96 h after infection and the difference in the CBP and p300 staining pattern (C, G). In the small cells the CBP staining in the nucleoplasm is trabecular, and in the blasts it is more homogeneous. Compared to the small cells, the intensity of p300 staining is stronger in the blasts. Scale bar, 5 μm.

Expression of EBNA-5 and CBP/p300 in EBV-infected B cells

In order to study the spatial relationship between EBNA-5 and CBP/p300, the cells were stained simultaneously with the anti-EBNA-5 and the anti-CBP/p300 reagents. In addition to the diffuse staining of the nucleoplasm, CBP and EBNA-5 appeared in brilliant nuclear dots (Fig. 3), identified as PML bodies (Fig. 4) (Székely *et al.,* 1996). All cells in the population were CBP positive and in those which expressed EBNA-5 the stainings in the PML bodies also completely overlapped (Figs. 3A–3C). In the nucleoplasm the CBP and EBNA-5 staining patterns were similar, but the degree of overlap could not be decided. All cells expressed p300. The PML bodies

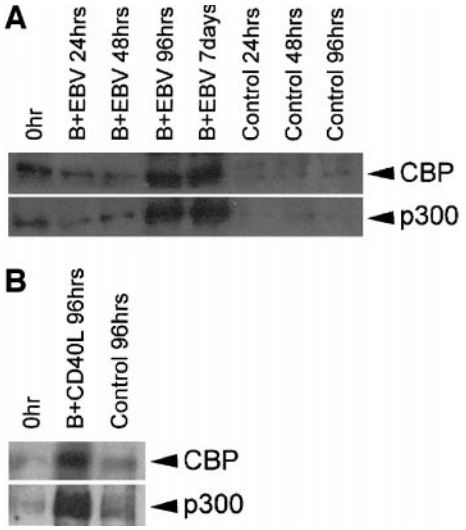


FIG. 2. (A) Western blot analysis for CBP/p300 in EBV-infected B cell populations at 0, 24, 48, and 96 h and 7 days of culture of B cells without and with EBV infection. (B) Western blot analysis for CBP/p300 in B cell populations stimulated by CD40 ligation at 0 and 96 h of coculture of B cells with irradiated CD40L-L cells or L cells (control).

did not stain for p300 and the patterns of EBNA-5 and p300 in the nucleoplasm differed. Thus the p300 and EBNA-5 proteins were differently located (Figs. 3D–3F).

Expression of EBNA-2, RBP-Jk, and CBP/p300 in EBV-infected B cells

The staining of EBNA-2 showed a speckled pattern. It overlapped considerably with the staining for RBP-Jk but not with that of CBP (Fig. 5). The stainings for EBNA-2 and p300 overlapped in some dots, which could be observed only in the 3D microscope (Fig. 6).

Expression of CBP and EBNA-5 in DG75 cells transfected with EBNA-5

EBNA-5 introduced by transfection into EBV-negative cells, DG75, was found to be localized in the PML bodies. The culture contained fewer than 5% EBNA-5-positive cells. In these cells, the staining for CBP and EBNA-5 overlapped completely (Fig. 7). On the other hand, DG75 cells transfected with EBNA-2 failed to show colocalization between EBNA-2 and p300. This culture contained many EBNA-2-positive cells (not shown).

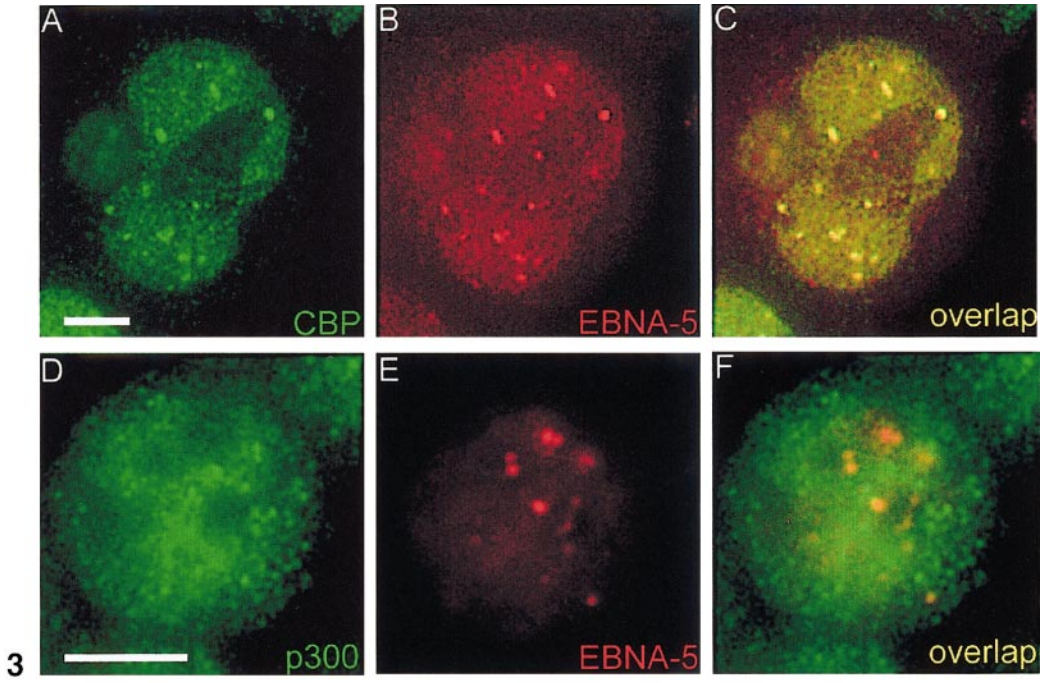
DISCUSSION

Growth transformation of B lymphocytes by EBV involves complex interactions between several virally encoded and cellular proteins. Among nine virally encoded proteins expressed in EBV-immortalized B lymphocytes EBNA-2 and EBNA-5 are the first to appear after *in vitro* infection. Positive cells can be detected after 12 h in the population (Alfieri *et al.,* 1991). EBNA-2 is a transcriptional activator and acts on specific promoters. The induction of LMP-1 expression by EBNA-2 seems to be the pivotal step for the acquisition of the immortalized phenotype. EBNA-2-induced LMP-1 activation requires its interaction with the cellular proteins RBP-Jk and PU.1, which bind to DNA (Henkel *et al.,* 1994; Johannsen *et al.,* 1995). EBNA-5 cooperates in this process through its enhancing effect (Harada and Kieff, 1997; Nitsche *et al.,* 1997).

CBP and p300 are related proteins and are usually mentioned together in discussions concerning their expression patterns and function. They belong to a family of transcriptional coactivators. Through interaction with a variety of cellular and viral proteins they can influence cell growth, transformation, and development (Goodman and Smolik, 2000). Their most important activity seems to be histone acetylation and they contain similar sequences within the HAT domains. Depending on the interacting protein partners, they may have opposite effects. Interaction of adenovirus-encoded oncoprotein E1A with CBP/p300 is thought to be critical for the transforming capacity of the virus. Chromosomal translocations, which affect CBP and p300, have been encountered in human malignancy (Goodman and Smolik, 2000).

In the present work, we have found that the elevation of CBP/p300 was induced by EBV infection (Fig. 2A). The time kinetics suggest that the elevation of CBP/p300 is not induced directly by EBNA-2 and EBNA-5 because these EBV-encoded proteins already appeared 24 h after infection. Indeed we found that CD40 ligation for 96 h also elevated CBP/p300 expression drastically (Fig. 2B). Taken together, the data suggested that CBP/p300 induction by EBV is related to the virus-induced activation and proliferation of the cells.

Recently, it has been shown that the activation of the LMP-1 promoter by EBNA-2 involves the contribution of CBP, p300, and PCAF (Wang *et al.,* 2000). In Akata cells EBNA-2-induced LMP-1 expression was upregulated by these coactivators. They bind to the acidic domains of EBNA-2. In 293T cells in which EBNA-2 and p300 were overexpressed by transfection, about 15% of the EBNA-2 was bound to p300. In our experiments with the EBV-infected B lymphocytes, some large dots showed colocalization of EBNA-2 and p300. However, EBNA-2-transfected DG75 cells failed to show colocalization with p300. This finding may suggest that cellular and/or viral factors that are present only in EBV-infected B cells are involved in the interaction between EBNA-2 and p300. RBP-Jk colocalized clearly with EBNA-2. RBP-Jk can have a repressive effect by binding to CBF1 interacting corepressor (CIR), which associates with the histone deacetylase complex (Hsieh *et al.,* 1999). EBNA-2 has been shown to block this function of RBP-Jk (Hsieh and Hayward, 1995). The almost complete colocalization of these two proteins suggests that EBNA-2 sequesters RBP-Jk. Jayachandra *et al.* (1999) showed that CBP and p300 bound to EBNA-2 in cotransfected mouse embryo



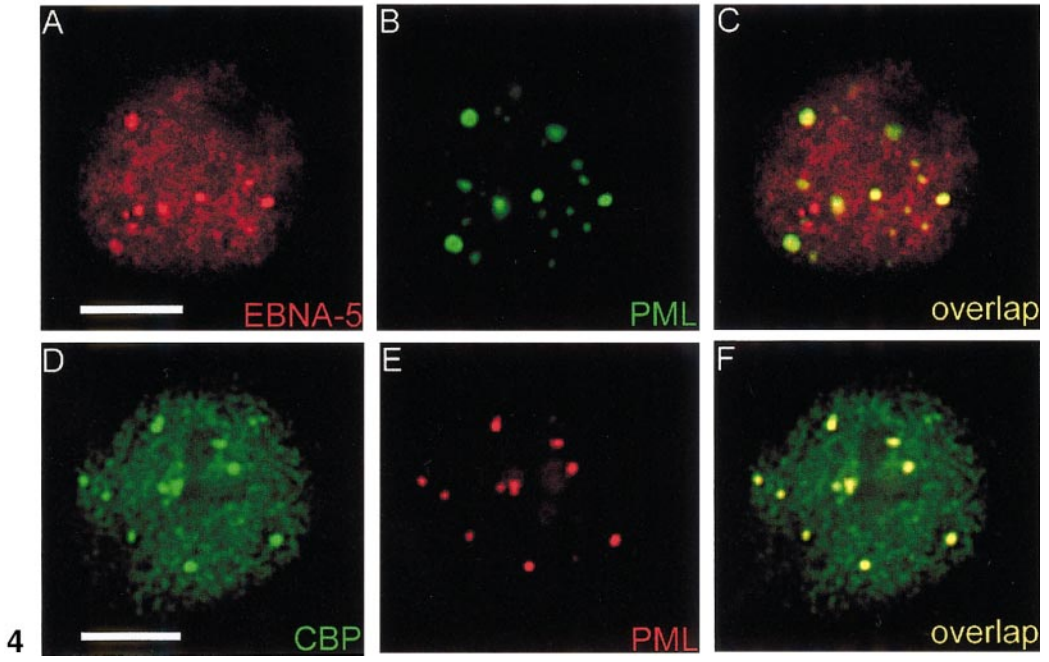


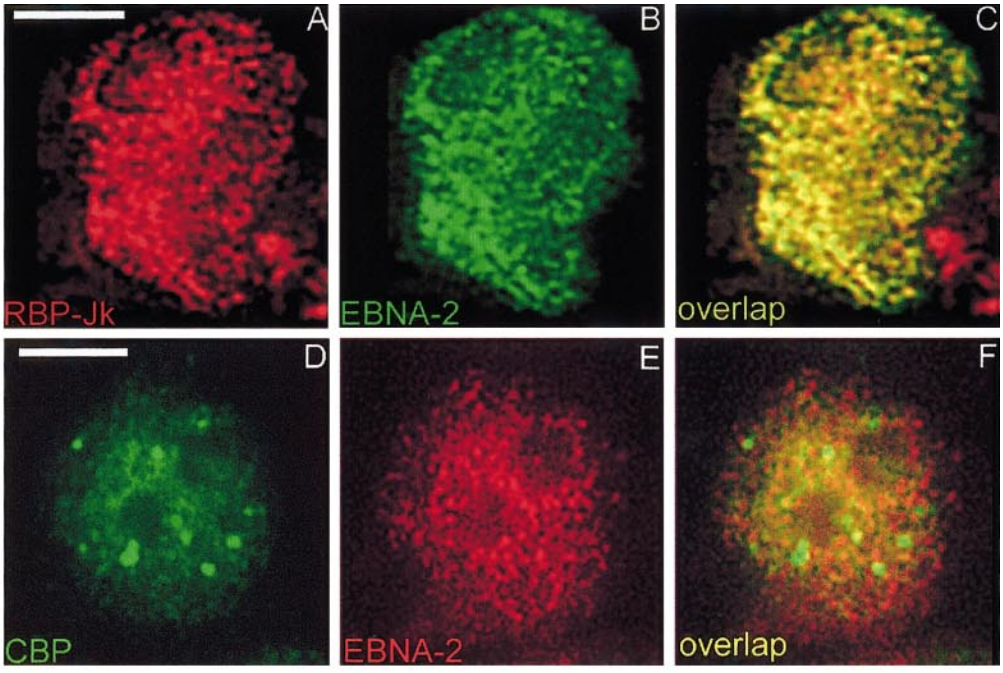
FIG. 3. Double immunofluorescence staining for EBNA-5 and CBP/p300 detection in B cells from a population 7 days after EBV infection. CBP/p300, green; EBNA-5, red. The population contained about 80% EBNA-5-positive cells. Note the CBP and EBNA-5 staining (A, B) in the large bodies. In addition, CBP and EBNA-5 (A–C) but not p300 and EBNA-5 (D–F) may colocalize in the nucleoplasm. Scale bar, 5 μm.

FIG. 4. Double immunofluorescence staining for EBNA-5 and PML, CBP, and PML in B cells 7 days after EBV infection. (A–C) EBNA-5, red; PML, green. (D–F) CBP, green; PML, red. Note that both EBNA-5 and CBP are expressed in the PML bodies. A complete overlap of CBP and PML is discernable, while EBNA-5 and PML may colocalize but not overlap. Scale bar, 5 μm.

FIG. 5. Double immunofluorescence staining for RBP-Jk and EBNA-2; for CBP/p300 and EBNA-2 in a population 7 days after EBV infection. (A–C) RBP-Jk, red; EBNA-2, green. (D–F) CBP, green; EBNA-2, red. Note the substantial overlap between RBP-Jk and EBNA-2 but no overlap between CBP and EBNA-2. Scale bar, 5 μm.

FIG. 6. Double immunofluorescence staining for p300 and EBNA-2 at low (A–C) and high (D–F) magnification in a population 7 days after EBV infection. P300, green; EBNA-2, red. Note that p300 and EBNA-2 colocalize in some large dots. These images were visualized by the 3D microscope using an optical deconvolution algorithm. Scale bar, 5 μm.

FIG. 7. Double immunofluorescence staining for CBP and EBNA-5 in DG75 cells with transfected EBNA-5. CBP, green; EBNA-5, red. Note the colocalization of CBP and EBNA-5 in the PML bodies. Scale bar, 5 μm.


A
RBP-Jk
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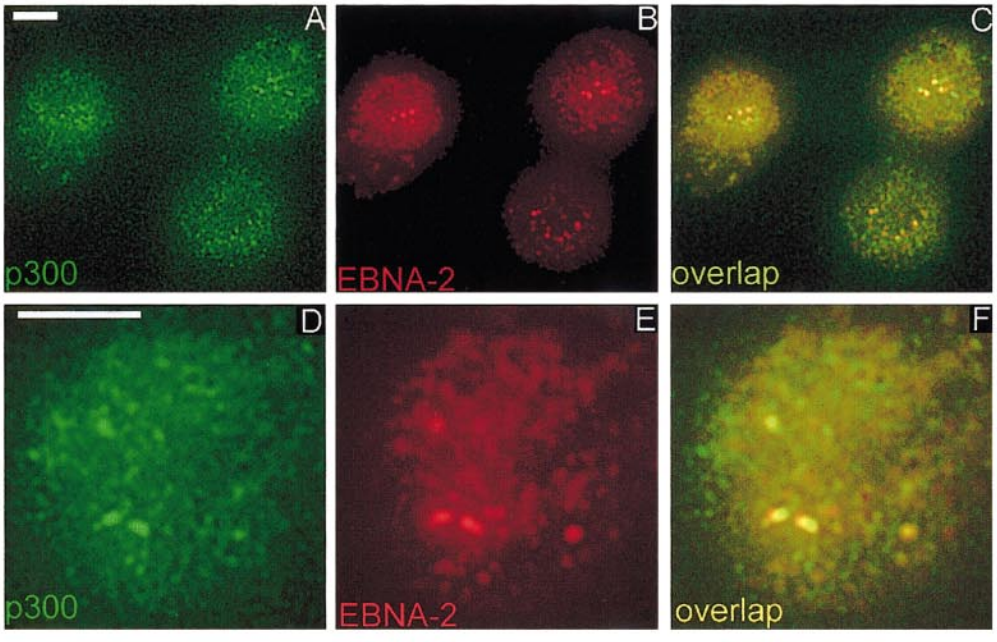
5


A
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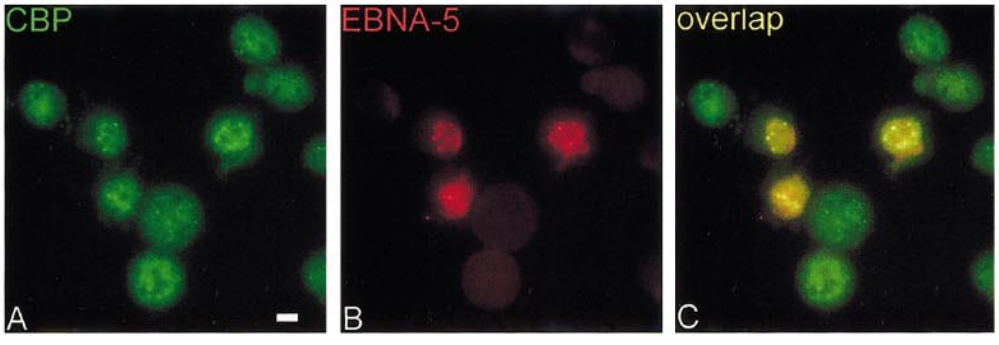
CBP
EBNA-5
overlap
A
B
C

7

fibroblasts. However, we could not detect colocalization of CBP and EBNA-2 in the B cells.

In contrast to the results of von Mikecz *et al.* (2000), who found p300 in a subset of PML bodies in Hep-2 cells, we could not detect p300 in the PODs of B lymphocytes. The results may be due to the use of different cell types in the two sets of experiments. In summary, we found a difference in the spatial expression of EBNA-2 and -5 in infected B lymphocytes. EBNA-5 but not EBNA-2 appeared in the PML bodies. Similarly CBP and p300 differed with regard to their compartmentalization in the PODs. Their different spatial association with either EBNA-2 or EBNA-5 and the difference in their appearance in the PODs may provide new clues concerning the distinct functions of CBP and p300. The significance of the localization in the PODs for the functions of the proteins Hsp70, p53, CBP, and EBNA-5 is not yet known. Results obtained with influenza nucleoprotein suggested that PODs are sites for proteasomal degradation of ubiquitinated proteins (Anton *et al.,* 1999). If this is the case, EBNA-5 may also participate in the regulation of proteasomal degradation.

MATERIAL AND METHODS

Cell lines

All cell lines were grown in RPMI culture medium supplemented with 10% heat-inactivated FCS, 100 U/ml of penicillin, and 100 U/ml of streptomycin. The cultures were split 1:10 every fourth day. Murine L cells (control) and L cells transfected with CD40L (CD40L-L cells) were kindly provided by Dr. P. Garrone (Schering Plough, Dardilly, France). EBNA-5-transfected DG75 cells (DG75-EBNA-5) and EBNA-2-transfected DG75 cells were kindly provided by Dr. Lars Rymo (Gothenburg, Sweden).

Collection and EBV infection of tonsil-derived B cells

Tonsil samples obtained from tonsillectomies were briefly exposed to ethanol, washed, and minced. The tissue was thereafter passed through a fine metal mesh. After two subsequent sheep red blood cell rosettings, the remaining T cells were depleted with CD2 Dynabeads (Dynal AS, Oslo, Norway). The B cells were incubated with B95-8 virus for 1 h, washed, and then cultured in RPMI 10% FCS.

Collection and CD40 stimulation of peripheral blood B cells

Peripheral blood mononuclear cells were isolated from buffy coats of healthy donors by Ficoll–Paque (Pharmacia, Upsala, Sweden) separation. B cells were separated by positive selection using CD19 Dynabeads M-450 (Dynal) followed by DETACHaBEAD CD19 (Dynal) treatment. Purified B cells were cocultured with irradiated (15,000 rad) CD40L-L cells or L cells. After 96 h culture, the B cells were harvested and then analyzed.

Antibodies

The following antibodies were used: anti-CBP rabbit polyclonal antibody (A-22; Santa Cruz), anti-p300 rabbit polyclonal antibody (N-15; Santa Cruz), anti-RBP-Jk rat monoclonal antibody (T6719; kindly provided by Dr. T. Honjo, Kyoto) (Hamaguchi *et al.,* 1992), anti-PML mouse monoclonal antibody (PG-M3; Santa Cruz), biotinylated anti-EBNA-5 mouse monoclonal antibody (JF186) (Finke *et al.,* 1987), anti-EBNA-2 mouse monoclonal antibody (PE-2; Dakopatts AB), swine anti-rabbit IgG-FITC (Dakopatts AB), goat anti-mouse IgG-biotin (Southern Biotechnology, Birmingham, AL), rabbit anti-mouse Ig-FITC (Dakopatts AB), rabbit anti-rat absorbed against mouse Ig (Dakopatts, Glustrup, Denmark), and Texas red-conjugated swine anti-rabbit Ig (Dakopatts).

Immunofluorescence

The cells were deposited on slides with cytospin and fixed in −20°C acetone–methanol (1:1). The slides were exposed for 20 min to 10% normal goat serum for blocking and thereafter incubated with the first antibody, A-22 anti-CBP (1:400) or N-15 anti-p300 (1:100), for 60 min at room temperature. This was followed by 30 min incubation with swine anti-rabbit IgG-FITC (1:40). Nuclear DNA was stained with 40 ng/ml of Hoechst 33258 (Molecular Probes, Europe BV). The stained cells were mounted with 70% glycerol, 2.5% DABCO (Sigma), pH 8.5, in PBS.

Double immunofluorescence stainings for CBP or p300 and EBNA5 were carried out in the following order: 10% normal goat serum, A-22 anti-CBP antibody (1:400) or N-15 anti-p300 (1:100), swine anti-rabbit IgG-FITC (1:40), 10% normal mouse serum, JF186 biotinylated anti-EBNA-5 (1:30), and Texas red-conjugated streptavidin (1:250, Vector Laboratories, Burlingame, CA). Double immunofluorescence for CBP or p300 and EBNA2 was carried out in the following order: 10% normal goat serum, A-22 anti-CBP antibody (1:400) or N-15 anti-p300 (1:100), swine anti-rabbit IgG-FITC (1:40), PE-2 anti EBNA-2 (1:20), goat anti-mouse IgG-biotin (1:100), and Texas red-conjugated streptavidin (1:250).

Double staining for PML and CBP was carried out in the following order: 10% normal goat serum, A-22 anti-CBP antibody (1:400), swine anti-rabbit IgG-FITC (1:40), PG-M3 anti-PML (1:50), goat anti-mouse IgG-biotin (1:100), and Texas red-conjugated streptavidin (1:250). Double PML and EBNA5 was carried out in the following order: 10% normal rabbit serum, PG-M3 anti-PML (1:50), and rabbit anti-mouse Ig-FITC (1:20), 10% normal mouse serum, JF186 biotinylated anti-EBNA-5 (1:30), and Texas red-conjugated streptavidin (1:250).

Double staining for EBNA-2 and RBP-Jk was carried out as follows: First, the EBNA-2 specific antibody PE-2

(1:20) was used. This mouse Ig was detected with goat anti-mouse IgG–biotin (1:100). Excess anti-mouse Ig reactivities were eliminated by 10% mouse serum. The cells were then incubated with rat monoclonal anti-RBP-Jk T6719 (1:50)/10% mouse sera overnight at 4°C. The rat Ig was probed with rabbit anti-rat absorbed against mouse Ig (1:30). Then biotin and rabbit Ig were detected by the mixture of FITC-conjugated streptavidin (1:250, Pharmingen, San Diego, CA) and Texas red-conjugated swine anti-rabbit Ig (1:30).

Image analysis

The slides were examined in a Leitz DM RB microscope. Composite filter cubes were used for the FITC, Texas red, and Hoechst 33258, respectively. The images were captured with a HAMAMATSU dual mode cold-CCD camera (C4880) as 8-bit uncompressed TIFF files. They were analyzed using Image-Pro Plus (Media Cybernetics) and Adobe PhotoShop 3.0 (Adobe Systems Inc.) as previously described (Teramoto *et al.,* 2000). Some of the images (Fig. 5) were captured on a 3D microscope using the ST-IFH-bind program that is a novel derivative of the previously published TROOPER3 and STEREOTROOPER with correlation for shine through (Holmvall and Székely, 1999). This program creates a reconstituted image from a series of optical sections (at 0.2 μm distance) taken by cold-CCD camera with wide-field illumination. The sections were deblurred using the nearest neighbor algorithm.

Immunoblotting

The cells were lysed in loading buffer. Aliqouts corresponding to 2.5×10^5 cells were electrophoresed in 5% SDS–PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes at 75V for 2 h. After blocking the membranes by exposure to 7.5% nonfat dried milk in PBS–Tween 20 for 1 h, they were incubated with the anti-CBP/p300 antibody overnight at 4°C. The blots were then incubated with horseradish peroxidase-conjugated donkey anti-rabbit Ig antibody (Amersham, Arlington Heights, IL, 1:2500 dilution) and detected with ECL+Plus detection reagent (Amersham).

ACKNOWLEDGMENTS

We thank Karin Kvarnung for excellent assistance. This work was supported by the Swedish Cancer Society (Cancerfonden). Kentaro Bandobashi, Akihiko Maeda, Norihiro Teramoto, and Noémi Nagy are recipients of fellowships from the Concern Foundation in Los Angeles and the Cancer Research Institute in New York.

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